

SYNTHETIC BIOMIMETIC FLUOROPHORES FOR MICRO/NANOSENSOR

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Abstract

Proteins common in nature provide a rich source of potential fluorophores that can be used as taggant materials. Many of these fluorophores have been demonstrated to be effective molecular probes (e.g., green fluorescent protein (GFP)). The objective of this research is to find, isolate, sequence, and synthetically produce (from a natural source) synthetic fluorophores extracted from reef coral or rcGFP. From this investigation we discovered two novel clones with unique sequences for rcGFP and rcCFP. This research provided a better understanding of rcGFP's suitability for incorporation into micro or nano-sensory devices. Once synthetically created, these fluorophores are meant for design as triggering and signaling devices. These optical switches will possess far greater environmental stability and survivability than their natural analogs. Micro and nano-sensory devices are envisioned as helping the soldier to better understand the battlespace environment by providing remotely sensed, geospatial awareness.

1. INTRODUCTION

Almost everything that occurs in the cell involves one or more proteins. Proteins provide structure, catalyze cellular reactions, and carry out a myriad of other tasks. Their central place in the cell is reflected in the fact that genetic information is ultimately expressed as protein. For each protein there is a segment of deoxyribonucleic acid (DNA), a gene, which encodes information specifying its sequence of amino acids. There are thousands of different kinds of proteins in a typical cell, each encoded by a gene and each performing a specific function. Proteins are among the most abundant biological macromolecules and are also

extremely versatile in their functions (Lehninger et al. 1993).

Proteins are classified according to their biological function. The most varied and most highly specialized proteins are those with catalytic activity—the enzymes. Transport proteins in blood plasma bind and carry specific molecules or ions from one organ to another. Other proteins are specialized for nutrients, storage, contraction, movement, structure, defense, and regulatory functions. There are numerous other proteins whose functions are rather exotic and not easily classified. For example, the blood plasma of some Antarctic fish contains antifreeze proteins, which protect their blood from freezing (Lehninger et al. 1993). Another exotic protein, which has sparked curiosity throughout the biotechnological community the past few decades, is GFP and GFP-like proteins. GFP has been utilized as a biosensor in many molecular and cellular biology applications (Cormack 1998, Czymmek et al. 2002, Howard et al. 2002, Smith et al. 2002). Recently, GFP-like proteins have been discovered in zooxanthellate corals and the function of these proteins is yet to be fully explained as seen in the literature (Mazel 1995, Dove et al. 1995, Takabayashi et al. 1995).

Czeczuga (1983) reported that the vivid colors of some reef-building corals and other invertebrates are among the most conspicuous elements of living coral reefs. With this in mind, it is perhaps surprising that so little is known about the identity and role of the various pigments in reef-associated organisms. Twenty years later, Dove et al. (2000) recognized that little is known about the structure and function of the pigments in the tissue of host corals.

The pigmentation of reef-building corals occurs in the skeleton of some species and in the epidermal and gastrodermal tissues of others (Kawaguti 1944, Takabayashi and Hoegh-

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Guldberg 1995). The chemical compounds responsible for coral pigments are known only in a few cases. For instance, proteins associated with some hydrocoral and scleractinian coral skeletons have been identified as carotin-protein complexes, but have not been identified to specific identifiable compounds (Fox and Wilkie 1970, Fox 1972, Ronneberg et al. 1979). Red and green carotin-protein complexes are also present in tissues of askeletal cnidarians such as *Actinia equina* and *Epiactis prolifera* (Czeczuga 1983). Among the most prominent pigments associated with the tissues of corals are the pinky-mauve pigments that are typical of the Pocilloporidae, Acroporidae, Poritidae, Fungiidae, and Maeruliniidae (5 out of the 16 families of reef-building corals) (Veron 1986). Although several skeletal pigments have been purified and identified, the function of the pigments in corals remains unexplored (Dove et al. 1995).

Stony corals (Scleractinia) of the class Anthozoa contain bright colors due to the fluorescent proteins of animal origin, many of which are intensely fluorescent under ultraviolet-A (UVA) and blue light absorptions with emission maxima at 420-620 nm (Salih et al. 2000). The Indo-Pacific coral *P. damicornis* has fluorescent proteins, which absorb at 496 nm and emit at 508 nm within the green region (490-520 nm) (Salih et al. 2000). In addition, a yellow fluorescent protein emits at 575 nm in *Porites cylindrica* (Salih et al. 2000).

Green fluorescent protein (GFP) was discovered by Shimomura et al. (1962) as a companion protein to aequorin, the famous chemiluminescent protein from the brightly luminescent *Aequorea* jellyfish, with glowing points around the margin of the umbrella. It has also been reported that this section of the umbrella contains the photogenic organ, which is where the emission of the GFP is derived (Shimomura 1979). This research group published the emission spectrum of GFP, which peaked at 508 nm. They noted that the green bioluminescence of living *Aequorea* tissue also peaked near this wavelength, whereas the chemiluminescence of pure aequorin was blue and peaked near 470 nm, which was close to one of the excitation peaks of GFP. Aequorin, isolated from the jellyfish *Aequorea victoria*, is a complex of the luciferin derivative coelenterazine, molecular oxygen and apoaquorin (apoprotein). If activated by Ca^{2+} binding it catalyses the oxidation of

coelenterazine to the excited state coelenteramide. When coelenteramide returns to its ground state, blue light at 470 nm is emitted (Niwa et al. 1996). The GFP converted the blue emission of aequorin to the green glow of the intact cells and animals (Tsien 1998).

Morin and Hastings (1971) found the same color shift in the related coelenterates *Obelia* (a hydroid) and *Renilla* (a sea pansy) and were the first to suggest radiation-less energy transfer as the mechanism for exciting coelenterate GFPs *in vivo*.

The cytoplasm of photogenic cells within the yellow tissue masses is densely packed with fine granules that contain the components necessary for bioluminescence in *A. victoria* (Jefferson et al. 1987, Davenport 1955). In other bioluminescent coelenterates these have been characterized as 0.2 micrometer-diameter particles enclosed by a unit membrane, and have been termed lumisomes (Anderson et al. 1973).

Prasher et al. (1992), Cody et al. (1993), Heim et al. (1994) have observed that the intrinsic fluorescence of GFP is due to a unique covalently attached chromophore complex, which is formed post-translationally within the protein upon cyclisation and oxidation of residues 65-76, Serine (Ser)-Tyrosine (Tyr)-Glycine (Gly). GFP is made up of 238 amino acid residues in a single polypeptide chain and emits a greenish fluorescence when irradiated with long ultraviolet light (Niwa et al. 1996). Morise et al. (1974) purified and crystallized GFP, measured its absorbance spectrum and fluorescence quantum yield, and showed that aequorin could efficiently transfer its luminescence energy to GFP when the two were co-adsorbed onto a cationic support. Prendergast and Mann (1978) obtained the first clear estimate for the GFP monomer molecular weight. Shimomura (1979) proteolyzed denatured GFP, analyzed the peptide that retained visible absorbance, and correctly proposed that the chromophore is a 4-(*p*-hydroxybenzylidene)-imidazolidin-5-one attached to the peptide backbone through the 1- and 2-positions of the ring (Tsien 1998).

GFP-like proteins are a family of homologous 25-30 kDa polypeptides that, together with *Aequorea victoria* GFP mutants, cover the emission range from 442-645 nm. In comparison to other natural pigments (Johnson et al. 1962), GFP's strength lies in small size, better stability, relative ease of use and it can form internal chromophores without requiring any accessory factors other than molecular oxygen

(Chalfie 1995). GFP has been extensively used in environmental biotechnology (Prasher et al. 1992) in the past. For this research we identified and extracted suitable, high quantum yield fluorescent proteins from the Horn coral, *Hydnophora rigida*.

The sequence and expression of rcGFP was studied in various reef corals to investigate their use in micro / nanosensor fabrications as photo-differential detectors. Current studies do show that reef coral proteins have been found to express their fluorescence due to constitutive fluorescent proteins. Fluorescent proteins are part of a group of coral pigments. This group includes brightly colored, low fluorescent forms and the highly fluorescent forms (Dove et al. 2000). Both types of proteins are partially homologous to green fluorescent protein (GFP) (Matz et al. 1999, Dove et al. 2000), first found in the luminescent jellyfish *Aequorea* and used in many molecular and cellular biology applications (Cormack 1998, Czymbek et al. 2002, Howard et al. 2002, and Smith et al. 2002).

To yield a synthetic product for this study, we used full length mRNA or DNA sequences of the fluorescent protein, which were amplified out of the library using gel-purified degenerative primers made to the first ten amino acids at the 5' end or N-terminus of the most common sequence. This yielded the synthesized product. Once the fluorophore was successfully synthesized, it was tested against its natural analog to aid in the understanding for a fluorescent molecular marker. Baseline steady-state measurements were also measured.

2. METHODS AND PROCEDURES

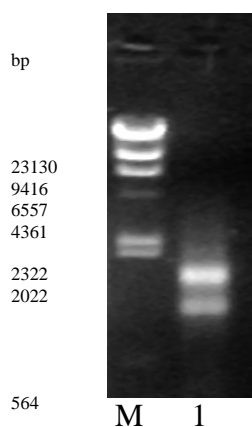
2.1 Collection and preparation of coral tissue samples:

Colonies of corals (*Heliopora coerulea*, *Caulastrea echinulata*, *Zooanthid* sp. and *Hydnophora rigida*) were purchased at a local marine aquarium (Herndon, VA) and kept frozen (-70°C), till the day of processing. Only fraction of the tissue was used for RNA isolation. In brief, small piece of pre-weighed frozen tissues were grinded manually in clean pre-chilled mortar and pestle (free of RNase's) without thawing.

2.2 Isolation of RNA:

The Ambion totally RNA isolation kit was used to isolate RNA. Once the tissue was

grounded to slurry it was mixed with 10 volumes of denaturation solution (Ambion). Lysate was then centrifuged at top speed at 4°C for 2-3 min. to remove any debris or insoluble material to desired number of microcentrifuge tubes. At this stage the volume of the lysate (*starting volume*) was noted in each tube, leaving the capacity slightly more than twice the volume of lysate. To the lysate 1 starting volume of Phenol: Chloroform:IAA (organic phase, under thin upper layer of aqueous buffer) was added and vortexed it vigorously for 1 minute. Solution was stored on ice for 5 min and then centrifuged at 4°C for 5 min at 10,000-x g. Aqueous phase was separated carefully without disturbing the interface of the layer. To the phenol extracted aqueous phase, 1/10 volume from aqueous phase of Sodium acetate solution was added and mixed by shaking or inverting for about 10 sec in RNase free microcentrifuge tubes. Lysate obtained in previous step was mixed with 1 *starting volume* of organic phase Acid-Phenol: Chloroform by vortexing for 1 min. The lysate kept on ice for 5 min and centrifuged at 10,000-x g and the upper aqueous phase obtained was transferred to a new RNase-free tubes with a capacity of at least twice the volume of the lysate at this point without disturbing the organic phase. To the aqueous phase obtained equal volume of isopropanol was added and mixed well and kept the preparation at -20°C for at least 30 min. Supernatant was carefully removed with the help of fine pipette without disturbing the pellet and the tube was briefly centrifuged to remove any residual fluid. The RNA pellet obtained was washed with 75% room temperature ethanol by flicking the tube with fingers for 2 min. RNA was isolated by centrifugation at ~7500 rpm for 10 minutes at 4°C. Ethanol was carefully removed without disturbing pellet with fine pipette and pellet was resuspended in the desired volume of DEPC water/EDTA. Total RNA obtained was treated with DNaseI for 30 min at 37°C. 10 µl aliquots of RNA were for stored at -80°C. RNA obtained was quantitated and its purity was checked by measuring the ratio A260/A280 (Fig. 1).



M = Marker
1 = RNA sample

Fig. 1: RNA sample.

2.3 cDNA PREPARATION:

Small-scale protocol from Ambion RLM-RACE kit was followed to prepare the cDNA from total RNA. In short 1 µg of total RNA was treated with calf intestine alkaline phosphatase (CIP) for 1 h and reaction was terminated by adding Ammonium acetate solution and acid phenol chloroform. The tube was vortexed thoroughly, centrifuged for 5 min at RT at 10,000 x g and aqueous phase (top layer) was transferred to a new tube. For the aqueous phase 150 µl chloroform was added, vortexed thoroughly, centrifuged for 5 min at RT at ≥10,000 g and the aqueous phase was transferred to a new tube. To the aqueous layer 150 µl isopropanol was added, vortexed thoroughly, chilled on ice for 10 min. and centrifuged at 10,000 x g for 20 min. at 4°C. The pellet was rinsed with 0.5 ml cold 70% ethanol, centrifuge for 5 min at 10,000 x g, removed ethanol carefully and allowed pellet to air dry. The RNA was resuspended in tobacco acid pyrophosphatase (TAP) buffer and stored at -20°C overnight.

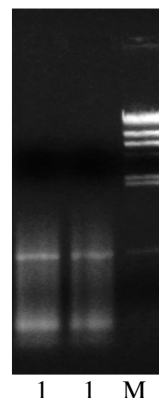
The RNA later on was treated with tobacco acid pyrophosphatase at 37°C for 1 hour. The TAP treated RNA was ligated with the 5'-RACE adaptor (5'-GCUGAUGGCGAUGAAUGAACACUGCGUUUGCUGGCUUUGAUGAAA-3') at 37°C for 1 hour and reverse transcription was carried out using polyT-RACE primer(5'-

GCGAGCACAGAATTAATAC
GACTCACTATAGG

TTTTTTTTTTTTTVN-3') at 42°C. The reaction was terminated by heating at 70°C for 10 min, chilled on ice for 2-3 min and treated with RNaseH for 20 min at 37°C.

2.4 Polymerase chain reaction

The PCR conditions were optimised, where necessary, according to the orthogonal array method (Cobb *et al.* 1994). The annealing and elongation thermal parameters were then adjusted to obtain optimal conditions. A Perkin Elmer thermal cycler was used for all reactions. The products of the PCR were stored at 4°C or used immediately (Fig. 2).



M = Marker
1 = GFP-PCR product from cDNA

Fig. 2: GFP-PCR product.

2.5 Primers

During primer design, care was taken to avoid potential internal secondary structure and, where possible, a GC clamp was engineered at the 3' end of the primer. Additionally, primers were checked to avoid overlap and possible dimerisation, potential for secondary structure formation and for compatible T_m values. T_m values were calculated according to the equation:

$$T_m (^{\circ}\text{C}) = 4(\text{G} + \text{C}) + 2(\text{A} + \text{T}) - 5^{\circ}\text{C}$$

Primers (50 nmol, desalted and deprotected) were obtained from Life technologies and resuspended in sterile distilled water to give final concentrations of 10 µM/µl for PCR. The nucleotide sequences were taken from various fluorescent gene sequences (Carter *et al.* 2004);

which included:

Primer 5'-RACE outer primer Forward 5'-3', sequence:
GCTGATGGCGATGAATGAACACTG;

Primer 3'-R1mc1 Reverse 3'-5', sequence:
CTTTTTGTGGTCTTGGCTTTTC
Position: 772-793
GAAAAGCCAAGACCACAAAAAG
(Clone name: mc1);

Primer 3' R1mc5 Reverse 3'-5'; sequence:
TCGTTTGGGCTTTTAGTTAAGC
Position: 762-783
GCTTAACTAAAAGCCCAAACGA
(Clone mc5);

Primer 3' R1zYFP Reverse 3'-5', sequence:

CTTATCAGGCCAAGGCAGAA
Position: 742-761
TTCTGCCTTGGCCTGATAAG
(Clone zoanYFP);
Primer 3' R1mcFP Reverse 3'-5', sequence:
CCTTAACCGGCAGCATAGAA
Position: 651-670
TTCTATGCTGCCGGTTAAGG (Clone mcFP);

Primer 3' R1mcavFP
Reverse 3'-5', sequence:
CGCAAATCTCAATGGAGTG
Position: 671-690
CACTCCATTGAGATTTTGCG (Clone
mcavFP_7.5) to look for similar homologs in
Hydnophora (Horn coral).
(Clone name: mc1);

2.6 Cloning protocol

2.6.1 DNA preparation

Amplified DNA was purified from the low melting agarose gel using the Qiagen gel extraction kit. After gel purification, the poly A was added to purified DNA in 20 µl reaction in a Perkin-Elmer thermocycler (72°C for 15 min). The resultant DNA was ligated with the TOPO-TA expression vector at RT for 20 min, according to manufacturer's instructions. The 2 µl of resultant plasmid was added to TOPO competent *E. coli* cells and let it sit on ice for 5 min. The cells were transformed by heat shock at 42°C for 30 sec and 500 µl of pre-warmed (37°C) SOC medium was added to the cells and then incubated in a water bath at 37°C with reduced shaking speed for 1 h and then plated on LB plate Ampicillin 100 µg/ml. The plates were incubated overnight at 37°C. Various transformant white colonies were picked and resuspended in TE and lysis was carried out at 95°C in thermocycler for 15 min. The lysed mixture was centrifuged and clear supernatant was used as template DNA in PCR (Fig. 3).

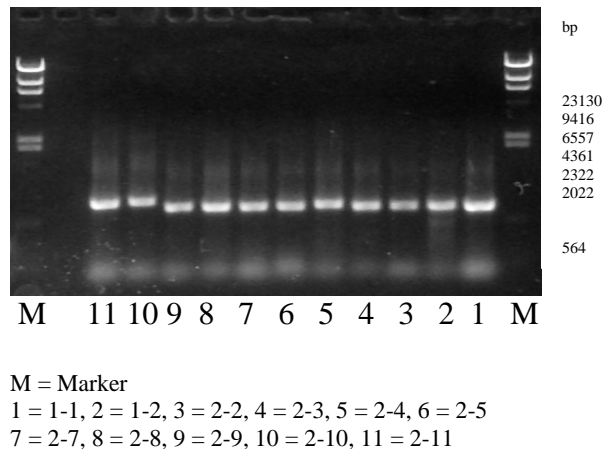


Fig. 3: Various rcGFP Clones

3. RESULTS AND DISCUSSION

3.1 Fluorescence Microscopy and Spectroscopy:

The successfully transformed colonies were seen under a fluorescent microscope initially; along with proper controls on slides (Fig.4). The cells grown overnight in LB media containing 30 µg Kanamycin were harvested and washed with a filter (0.2 µM) in sterile PBS. In addition emission and excitation spectra were measured.

rcGFP fluorescence (Clone 2-2)

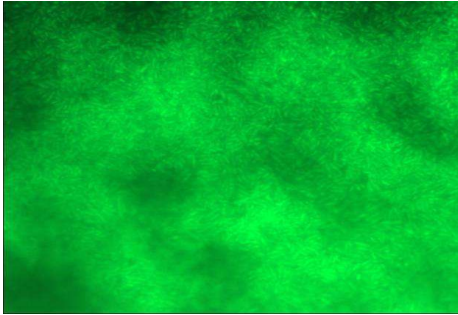


Fig. 4: Fluorescence microscopy of rcGFP Clone 2_2 with a GFP filter at 100 x (490 nm Ex and 510 nm Em).

Two types of clones from the Horn coral, *Hydnophora rigida* were observed. Clones 1-2, 2-2, 2-3, 2-4, 2-8, 2-11 were positive for a GFP-like protein (rcGFP) with one major excitation peak at 507 nm and emission peak at 527 nm. We called these clones rcGFPs (Fig. 5).

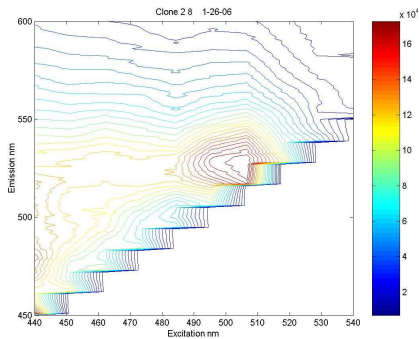


Fig. 5: Excitation emission matrices (EEM) of Clone 2-8 showing the green fluorescence peaking 507 nm excitation and 527 nm emission.

Clones: 1-1, 2-7, 2-9 had a blue or cyano-centroid with one major excitation peak at 450 nm and emission peak at 495 nm. We called these clones rcCFPs (Fig. 6).

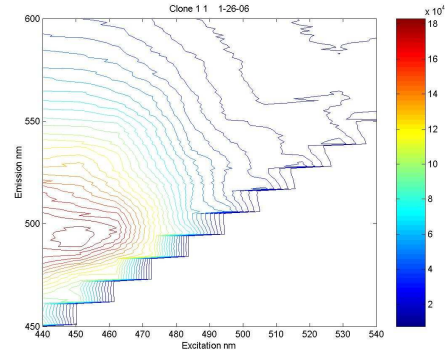


Fig. 6: Excitation emission matrices (EEM) of Clone 1-1 showing the cyano fluorescence peaking 450 nm excitation and 495 nm emission.

Bacillus subtilis endospores that express GFP, derived from the *Aequorea*, upon sporulation were used as a control as a known green fluorescence measurement to compare to the rcGFP clones derived from *Hydnophora* (Fig.7). The *Hydnophora* rcGFP excitation and emission data practically overlap with the *Aequorea* GFP excitation as shown by the EEMs.

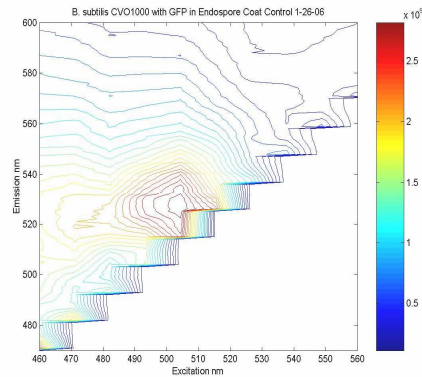


Fig. 7: Excitation emission matrices (EEM) of *B. subtilis* endospores expressing GFP upon sporulation.

CONCLUSIONS

Although the reading frames are short, there is no identifiable protein motif using BLASTX in any of the reading frames, which means these two clones have sequences that are novel with respect to fluorescent protein motifs. Sequencing was repeated to clarify the protein sequence and identify the fluorescent protein

motifs. Future efforts will involve the expression of these new fluorescent proteins in *Bacillus* endospores as labels for sporulation and potentially germination investigations. In addition, it may also provide new information as to whether the biotechnological world may utilize these fluorescent proteins as an alternative biological label for reporting the presence of harmful chemicals and/or biochemical constituents within the environment using remote imaging technology.

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